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A cleaved form of prolactin, named 16K PRL, has been reported to act as an angiostatic agent in several vascular beds. We found that rat mammary gland homogenates can proteolytically cleave PRL and generate 16K PRL. We also detected significant amounts of immunoreactive PRL in human breast tissue and determined that breast cancer cells express PRL receptors and have the capacity to internalize and then release exogenous PRL. Our working hypothesis is that mammary cells internalize PRL from the circulation by a receptor-mediated mechanism. Inside the cell, PRL is cleaved to yield 16K PRL, which is then released, binds to specific receptors on neighboring endothelial cells and suppress their proliferation. The process of tumorigenesis is associated with disruption in the formation or action of 16K PRL, thereby enabling angiogenesis and tumor expansion. Ongoing experiments will establish the PRL cleavage capability of human breast tissue, will investigate the presence of 16K PRL in normal and tumorous breast tissue, and will test the angiostatic actions of 16K PRL.

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FOREWORD

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Nira Ben-Jonath 10/8/97

PI - Signature Date

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INTRODUCTION:

The role of prolactin (PRL) as a mitogen in breast cancer remains unsettled. Earlier in this granting period we found that lactating rat mammary glands and nitrosomethylurea (NMU)-induced mammary tumors express PRL. Incubation of NMU tumor cells with PRL antisera inhibited cell proliferation, suggesting that PRL is an autocrine mitogen in these cells (1). However, in spite of numerous trials, we detected no *de novo* synthesis of PRL by MCF-7 or T47D breast cancer cells, as judged by RT-PCR and Northern analysis. Although these cells express PRL receptors, proliferation of human breast cancer cells was unaffected by exogenous PRL or PRL antiserum. Others also reported that PRL is weak, or inconsistent, mitogen in breast cancer (2). Yet, PRL receptors are present in over 50% of tumor biopsy, and immunoreactive PRL is detectable in normal human breast and carcinomas. This leaves the question of both the origin and functions of breast PRL unresolved.

Given the above findings, we have formulated a new hypothesis: mammary cells can internalize PRL from the blood by a receptor-mediated mechanism. Once inside the cell, PRL is proteolytically cleaved to yield a 16K fragment. Upon release from the cell, 16K PRL binds to unique 16K PRL receptors on neighboring endothelial cells and maintains them in a growth-arrested state. We postulate that disruption in the formation of 16K PRL or dysfunction of its receptor constitute part of the 'angiogenic switch' of tumorigenesis that alters the balance between angiogenic and angiostatic factors, enabling neovascularization and accelerated tumor growth. This hypothesis is based on the following rationale: one, angiogenesis is a critical step in breast carcinogenesis and metastasis; two, 16K PRL has been reported to suppresses angiogenesis *in vivo* and *in vitro*; three, the mammary gland has a high capacity for generating 16K PRL.

BODY:

16K PRL: structure and angiostatic actions

PRL is a single chain protein composed of four anti-parallel α helices with three disulfide bridges between residues 4-11, 58-174 and 191-199. As shown in fig 1, PRL is cleaved in two steps: first, cathepsin D removes a tripeptide between residues 145 and 149, forming 'cleaved' PRL. Next, the joining bridge is reduced to yield N-terminal 16K PRL and C-terminal 8K fragment (3). 16K PRL was reported to inhibit endothelial cell proliferation and to antagonize the stimulatory effects of basic FGF (bFGF) and vascular endothelial growth factor (VEGF) in a dose-dependent manner (4). This was specific to endothelial cells and was not mediated by the FGF receptor. The angiostatic actions of 16K PRL were demonstrated *in vitro*, using endothelial cells from bovine brain and human umbilical vein, and *in vivo*, using capillary formation in the chick embryo chorioallantoic membrane. High affinity, specific and saturable binding sites for 16K PRL were found in membranes from brain capillary endothelial cells; these sites were not competed by 23K PRL, growth hormone (GH) or bFGF (5). The exact identity of the receptor, however, is still unknown.

Immunoreactive prolactin in normal human breast and in carcinomas

We examined the presence of PRL in human breast tissues by immunocytochemistry, using polyclonal antibodies against human PRL. As shown in fig 2, PRL was detected in a lactating breast and in both grade I and grade III ductal carcinomas. As expected, PRL was localized in the cytoplasm of the epithelial cells and also appeared to be localized in migratory cells such as macrophages or lymphocytes. Northern analysis and *in situ* hybridization failed to detect mRNA for PRL in similar tissues (data not shown). Based on these results we concluded that most PRL in breast tissue comes from extramammary sources, likely via uptake from the blood. The precise nature of intramammary PRL (e.g., whether it represents an intact or a cleaved molecule) cannot be distinguished by immunocytochemistry using polyclonal antibodies since 23K PRL and 16K PRL share 100% sequence homology.

Generation of 16K PRL by mammary gland homogenates

It has been reported that the lactating rat mammary gland has the highest PRL cleaving activity of all tissues examined, including liver, pituitary and testes (6). The cleaving enzyme was identified as Cathepsin D, an estrogen-inducible, acid protease that is highly expressed in both the normal human breast and in breast cancer (7). Before exploring whether human breast cells can cleave PRL, we had to establish a method for assessing tissue cleaving capacity. As described by others (6), we prepared a 25,000xg pellet from lactating rat mammary homogenates. The pellet was ressuspended in citrate buffer, pH 3.4, and incubated with rat PRL for 2h; incubation with heat-inactivated pellet served as control. Fig 3 shows the generation of 16K PRL (and small amounts of 8K PRL) by the mammary homogenates, as resolved on reducing SDS-PAGE. Heat inactivation of the homogenate, as expected, abolished its PRL cleaving capability. Interestingly, this cleavage of PRL by the rat mammary tissue was species- specific since only rat PRL, but not human, ovine or bovine PRL was cleaved.

Proposed mechanism of PRL cleavage

Theoretically, PRL cleavage can occur by two mechanisms: one, by PRL binding to its receptor followed by internalization; two, by cathepsin D that is released into the extracellular space. We favor the first mechanism, based on an analogy with parathyroid hormone (PTH). PTH 1-84 is taken up by macrophages into endosomes, where it is cleaved by cathepsin D. Cleaved fragments, including the biologically active PTH 1-34, are rapidly secreted by the cells without delivery to lysosomes (8). Our objective was to determine whether human breast cancer cells can cleave PRL. For cleavage of PRL to occur by a receptor-mediated mechanism, the cells should express the PRL receptor. To determine whether this postulate is plausible, we isolated total RNA from MCF-7 and T47D breast cancer cells and from human pituitary, serving as a control. Approximately 5μg of RNA were reversed transcribed and then subjected to PCR, using specific primers for human PRL receptor as well as human PRL. Each PCR reaction also contained primers for ribosomal protein L19 (RPL19), serving as an internal control. As shown in fig 4, we found that MCF-7 and T47D cells express the PRL receptor but not PRL itself

Uptake and release on iodinated PRL by human breast cancer cells

In a recent preliminary experiment we tested whether T47D breast cancer cells can take up and then release PRL. Human PRL was iodinated by the lactoperoxidase method and then purified on a gel filtration column. The cells were precultured for 48h in serum-free media to eliminate exogenous PRL and then were incubated with iodinated hPRL at 4C for 60 min. After washing the unbound hormone, cells were warmed to 37C to initiate internalization. Media aliquots and cell extracts were taken at 0, 30 and 60 min and counted. As illustrated in fig 5, labeled PRL in the medium increased, whereas intracellular PRL decreased, over a period of 60 min. Although the nature of released PRL has not yet been determined, these results support the notion that human breast cancer cells have the capacity to internalize PRL and then release either an intact, or a cleaved, product into the medium.

CONCLUSIONS

Our results demonstrate that human breast tissues contain significant amounts of immunoreactive PRL, most of which is not locally produced. The rat mammary gland has a high capacity for generating 16K PRL. Human breast cancer cells, MCF-7 and T47D, express PRL receptors and are capable of uptake, followed by release, of exogenous hPRL. Since 16K PRL was reported to act as an angiostatic agent, these preliminary results support our working hypothesis that 16K PRL may be important in controlling mammary angiogenesis.

The mechanism that normally maintains the endothelium quiescent but facilitates growth upon demand is enigmatic. The current view is that a fine balance exists between angiogenic and angiostatic factors and its disturbance causes neovascularization. Control of angiogenesis is likely tissue-specific, as suggested by the multitude of angiogenic and angiostatic factors (9). Clearly, ample evidence suggests that 16K PRL is angiostatic *in vivo* and *in vitro* and its action is conserved across species. The fact that a proteolytic fragment of a known protein possesses such activity is not unprecedented. Two of the most potent angiostatic factors, angiostatin and endostatin, are cleaved products of plasminogen and collagen XVIII, respectively. Potential effects of 16K PRL on breast vasculature have not been studied. Since 16K PRL lacks the fourth α helix, it assumes a different configuration than intact PRL, accounting for its poor binding to classical PRL receptors and weak mitogenic and lactogenic action on the mammary gland (10). To date, the receptor for 16K PRL has not been cloned and its tissue distribution is unknown.

If our hypothesis is correct, uptake, processing and release of 16K PRL is proportional to PRL receptor density in a given mammary cell. The implication for breast cancer is that PRL receptor mutation or downregulation may render tumor cells unable to generate 16K PRL, resulting in angiogenesis and tumor expansion. Alternatively, the process of tumorigenesis is associated with unresponsiveness of target endothelial cells to 16K PRL, resulting in removal of inhibition and accelerated neovascularization. There are several potential long term benefits from this research. One, measurement of 16K PRL in tumor biopsy, blood or urine may provide an independent prognosis for breast cancer (11). Two, 16K PRL could be used either alone, or in combination with chemotherapy or radiotherapy for targeting tumor vasculature and facilitating tumor shrinkage.

Because of this unexpected turn of our research, we have defined three new specific aims for the next year of funding: 1) to use uptake of labeled hPRL in combination with SDS-PAGE to determine whether breast cancer cells generate 16K PRL by a receptor-mediated mechanism; 2) to investigate whether the levels of 16K PRL in human breast carcinomas are inversely correlated with microvessel density; 3) to generate 16K hPRL by recombinant DNA technology and examine its angiostatic action using human umbilical vein endothelial cells (HUVEC).

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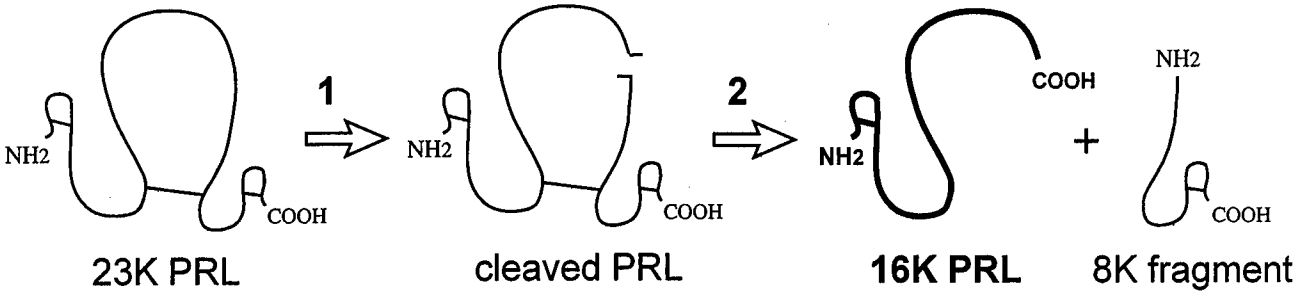


Fig 1: Formation of 16K PRL by a two-step process: (1) cleavage of 23K PRL by cathepsin D; (2) reduction of the disulfide bridge. Note the resulting change in the shape of the molecule.

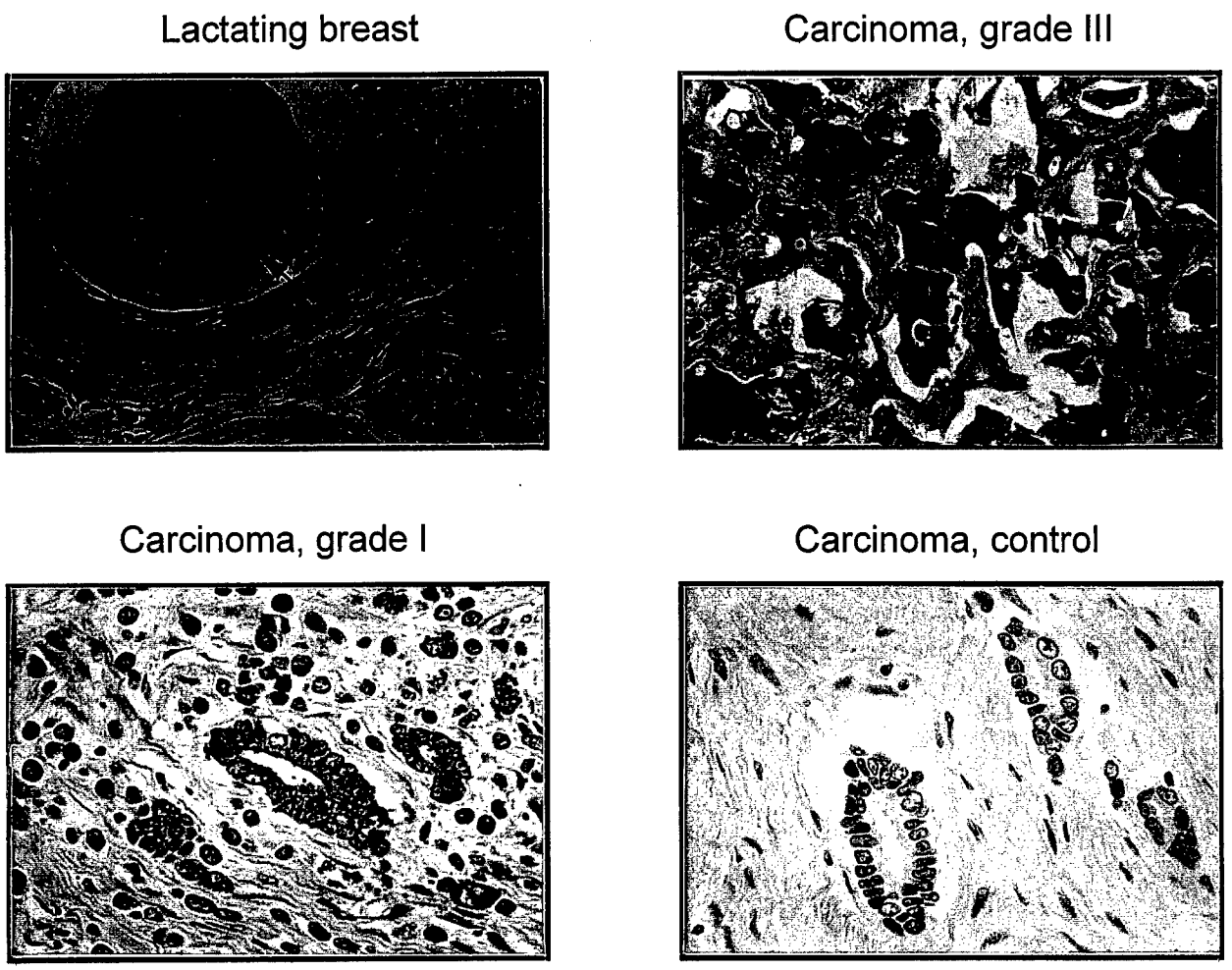
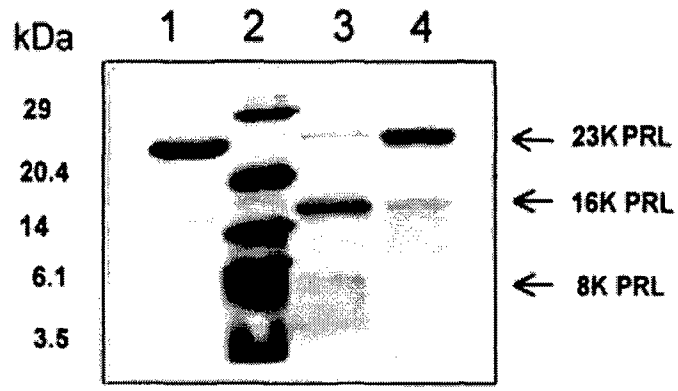


Fig 2: Immunoreactive PRL in a normal lactating breast (upper left), poorly differentiated (grade III) ductal carcinoma (upper right), and well differentiated (grade I) ductal carcinoma (lower left). All sections were counterstained with hematoxylin. Dilution of hPRL ab, 1:5000. The control section (lower right) had normal rabbit serum. Note the milk (arrow) in the alveolus of the lactating breast.

Fig 3: Generation of 16K PRL by the rat mammary gland. Rat PRL was incubated at pH 3.4, 37C for 2h with a 25,000xg pellet from lactating mammary homogenates. Aliquots were separated on reducing 10% SDS-PAGE and stained with Comassie Blue. Lane 1: intact rPRL; lane 2: MW standards: lane 3: rPRL incubated with homogenate; lane 4: rPRL incubated with heat-inactivated homogenate.



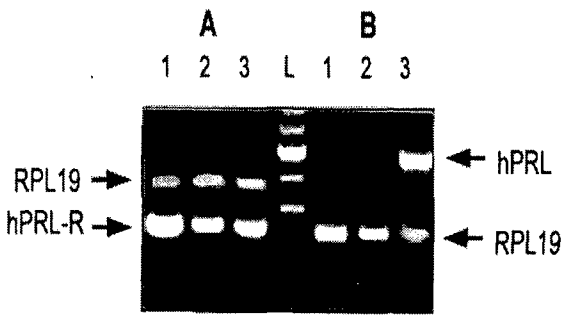


Fig 4: expression of PRL receptor (hPRL-R) but not PRL itself (hPRL) by human breast cancer cells, as determined by RT-PCR. In each panel, lanes 1,2 and 3 are T47D, MCF-7 and human pituitary, respectively. All primers are intron-spanning. hPRL-R: 350 bp; hPRL: 580 bp; RPL19 in panel A: 500bp; RPL19 in panel B: 333 bp; L: 100 bp ladder.

Fig 5: Uptake and subsequent release of iodinated hPRL by T47D breast cancer cells. The cells were incubated with ^{125}I-hPRL at 4C for 60 min. After washing the unbound hormone, cells were warmed to 37C and aliquots of media and cell extracts were taken for gamma counting. The nature of PRL (23K or 16K) has not yet been determined.